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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

Synthesis of the immune-specific, polypeptide-like hapten of the anthrax-subtilis bacilli group. A synthetic proof of the constitution of natural polyglutamic acids.

by V. Bruckner, M. Kajtar, J. Kovacs, H. Nagy and J. Wein
From the Organic Chemical Institute of Eotvos University, Budapest.

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Statement of Problem.

Some time ago Ivanovics and Bruckner (1) reported on the isolation of the immune-specific substance of the anthrax bacillus capsule and have shown that this substance represents a biuret-negative polypeptide which produces a high yield of D(-)glutamic acid-hydrochloride upon hydrolysis with hydrochloric acid. Simultaneously it was discovered that various gram positive, aerobic, mesophilic spore carriers (for example, B. subtilis, previously called B. mesentericus) excrete a substance in their nutrients which is easily isolated; it seemed to be identical with the specific capsule substance of anthrax bacilli (B. anthracis). Hereafter these two polypeptides will be referred to as anthrax-polypeptide (APP) and subtilis-polypeptide (SPP).

Later tests (2,3) have positively proven that both polypeptides are constructed of glutamic acid residues only, i.e. they represent monotonous polypeptides, as had been suspected following first examination (1,4). It has been proved that APP possesses a D-configurative uniformity (3,5) (consequently it is an optically pure poly-D-glutamic acid), while SPP, which can be isolated from the nutrient medium of B. subtilis grown in accordance with the original method (1), seems to contain about 85% D-isomeric and 15% L-isomeric glutamic acid residues (5). Recently it has even been discovered that the proportion of D and L-isomeric components of SPP is strongly de-

pendent on the method of cultivation of an identical strain of B. subtilis, so that the incorporated L-glutamic acid residues may in some cases amount to 80%. However, SPP preparations whose content of L-glutamic acid residues is strongly in excess of 15-20% have not yet been tested serologically. On the other hand, it has been discovered that SPP obtainable by means of the original method (1) shows as marked a serologic reaction as APP: Solutions of both polypeptides show a precipitating reaction (1) with anti-anthrax immune sera down to a dilution of 3.2×10^6. According to immune-biologic tests APP as well as SPP (obtainable by means of the original method (1) are to be considered immune-specific haptens (semi-antigens) of the anthrax-subtilis bacilli group.

It was inferrible from the results of a specific degradation to which APP and SPP were subjected that both polypeptides constitute gamma-poly-glutamic acids (III) (7). The reliability of this conclusion was first proved indirectly by the analogous degradation of synthetically prepared alpha-poly-L-glutamic acid-gamma-methylester and its D-antipode: alpha-gamma-diamino butyric acid (in the form of its diflavianate), which is the degradation product characteristic of the alpha-glutamyl bond, could be obtained, while beta-formyl propionic acid, the characteristic degradation product of the gamma-glutamyl bond, could be proved neither preparatively (for example as p-nitrophenyl hydrazone) nor chromatographically (8,9). Recently a direct proof of the usefulness of constitution-determining degradation was established, and this by means of the synthetically accessible (10) alpha gamma-poly-L-glutamic acid methylester, which is distinguished by constant alternation of alpha and gamma-glutamyl linkage in its peptide chain. In this case both of the expected degradation products were actually obtained preparatively.

It was decisive in constitution-determination of APP and SPP that the synthetic alpha-poly-glutamic acids be characteristically different from APP or SPP, not only by virtue of the result of the specific degradation, but also in respect to their properties (i.e. very poor solubility in water, positive biuret reaction, racemization in alkaline solution, no serologic reaction). This refutes the report of Hanby et al (3,11) that APP contained predominantly alpha-glutamyl bonds (and that the APP originally isolated (1) contained these exclusively). (12,13)

After first searching for the most suitable way in the L-series, the final step in constitutional proof has now been conducted: The synthesis of APP, i.e. of gamma-poly-D-glutamic acid (IIIb). Simultaneously with the last mentioned preparations and independently of us, Waley (14) also has accomplished the synthesis of gamma-poly-L-glutamic acid (IIIa). Waley used the same dipeptide derivative as key substance for the synthesis as we did (see below: L-L-start dipeptide; Ia); furthermore, essentially the same direction was taken in synthesizing the latter. Beside the gamma-poly-glutamic acid of the D and L series we have also synthesized gamma-poly-(gamma-L-glutamyl-D-glutamic acid)(IIIc). This type of stereo-isomeric gamma-poly-glutamic acid, which can simply be called mesoid gamma-poly-glutamic acid (IIIc), is distinguished by the fact that L and D-gamma-glutamyl residues constantly alternate in its peptide chain. This product was meant especially for comparison with SPP.

General plan of the syntheses.

The synthesis of above-mentioned three stereo-isomeric gamma-poly-glutamic acids were phased as follows. At first the gamma-glutamyl-glutamic-acid-alpha-alpha'-dimethylester (I) of the corresponding steric series was

cont. next page 4

synthesized, these three stereo-isomeric dipeptide derivatives are designated below as L-L-start dipeptide (Ia), D-D-start dipeptide (Ib) and L-D-start dipeptide (Ic), respectively. Then the bifunctional start dipeptides, by means of a previous activation of their carboxyl group or amino group, were subjected to intermolecular polyacylation, which led to formation of gamma-poly-glutamic acid-alpha-methylester (II). This conversion was accomplished in as concentrated a solution as possible, in order to minimize the possibility of formation of cyclopeptides. From the structural viewpoint it could be assumed that in the polyester the terminal carboxyl or amino group is still existant in its activated form (cf. for example the formulas XVI, XXI and XXX), however, in the course of production of free poly acids from corresponding polyesters such groups are again converted to free carboxyl or amino groups. In the production of free polyacid (III) the polyester was subjected to alkaline saponification, then the polyacid was separated first in form of its difficultly soluble copper(II)salt, liberated from the latter by hydrogen sulphide, purified by means of dialysis and thus freed of lower oligopeptides, and finally produced from the solution by freeze drying. It should be mentioned that polyesters in dimethyl-formamide solution associate strongly with this solvent, and are water soluble; this fact made possible the interpolation of an aqueous dialysis in connection with the polyester.

Synthesis of the stereo-isomeric start dipeptides.

We (12) have already given a full account (as has been done independently by Waley (14) of the synthesis of the L-L-start dipeptide, i.e. the gamma-L-glutamyl-L-glutamic acid-alpha alpha'-dimethylester (Ia). A few differing observations are given by way of the analogous synthesis of the D-D-start dipeptide (Ib).

The synthesis of the D-D-start dipeptide, i.e. of the gamma-D-glutamyl-D-glutamic acid-alpha alpha'-dimethylester (Ib), which was conducted in exact imitation of the synthesis of the L-L-start dipeptide (Ia), took the following course. Carbobenzoxy-D-glutamic acid-gamma-hydrazide (IV) was coupled via (non-isolated) azide (V) with D-glutamic acid-gamma-benzylester (VI) to carbobenzoxy-gamma-D-glutamyl-D-glutamic acid-gamma'-benzylester (VII). The directly resulting crystalline product was, according to expectations, contaminated with a little carbobenzoxy-alpha-D-glutayl-D-glutamic acid-gamma'-benzylester (IX), which could not be completely removed even by repeated recrystallization. This result, which naturally also applies to the totally identically synthesized L-L-product VII is in conflict with the observations made by Waley, who reported that upon reaction of L-acid azide V with the L-benzylester VI to form the derivative VII of the L-L-start dipeptide, the structure-isomeric L-L-derivative IX does not result simultaneously, or that it is not contained in the crystalline reaction product. In spite of the light contamination of product VII the synthesis was continued successfully. The next step was the coversion of product VII (in methanol solution) with diazomethane to carbobenzoxy-gamma-D-glutamyl-D-glutamic acid-alpha alpha'-dimethyl-gamma'-benzylester (VIII), which was obtained directly in crystalline form and which could very easily be recrystallized from methanol, whereby the conversion product of the structure-isomeric components, carbobenzoxy-alpha-D-glutamyl-D-glutamic acid-gamma alpha'-dimethyl-gamma'-benzylester (X), remained almost completely in the mother liquors. The hydrogenolysis (Pd-animal charcoal, methanol) of derivative VIII finally produced gamma-D-glutamyl-D-glutamic acid-alpha alpha'-dimethylester, i.e. the D-D-start dipeptide (Ib), whose paper chromatographic analysis showed that the admixture of structure-isomeric compound XI amounted to a maximum of 0.5%. The crystalline L-L-start

dipeptide (Ia) contains 1 mole of crystal water (12), as also found by Waley (14). We have determined, contrary to Waley, that the crystal water cannot be removed by ordinary drying (except perhaps in the vacuum pistol at a moderate temperature) without secondary transformation. Of course this also applies to the D-D-start dipeptide (Ib).

By the method described above the L-D-start dipeptide also was synthesized, i.e. gamma-L-glutamyl-D-glutamic acid-alpha alpha'-dimethylester (Ic); the first step consisted of converting the L-azide (V), which had been freshly prepared from carbobenzoxy-L-glutamic acid-gamma-hydrazide (IV), by means of D-glutamic acid-gamma-benzylester (VI) into carbobenzoxy-gamma-L-glutamyl-D-glutamic acid-gamma'-benzylester (VII). Again all intermediate products, with the exception of L-azide (V) which was not isolated, proved to be well-crystallizing compounds.

Conversion of the stereo-isomeric start dipeptides into gamma-poly-glutamic acid-alpha-methylesters (II), the corresponding stereo-isomeric forms.

Following conversion of the carboxyl or amino group into a reactive group, the intermolecular polyacylation of the bifunctional start dipeptide (I) could be accomplished easily, leading to the polyester (II). This activation, which in this case happened simultaneously with the intermolecular conversion, took place in four different ways (method 1-4) in respect to the L-L-start dipeptide (Ia). In connection with the more costly D-D-start dipeptide (Ib) and the L-D-start dipeptide (Ic) only two and one method, respectively, were used (methods 2 and 4, and method 2, respectively).

(1) The first method represents a commensurate alteration of a process which has already been stated: At first carbobenzoxy-gamma-L-glutamyl-L-glutamic acid-alpha alpha'-dimethylester (XII) was produced from the L-L-start dipeptide (Ia), then according to Boissonnas' method, converted to the mixed

ester anhydride XIII by reacting with chloroformic acid-ethylester in ice cold dimethyl formamide solution in the presence of 1 mole of triethylamine. The mixed ester anhydride, while in its ice cold solution, was subjected to hydrogenolysis following addition of Pd-animal charcoal. This process was not effected in a closed container, for reasons which have already been discussed elsewhere (12), but was conducted with a current of hydrogen. The yield of CO_2, which was determined in the escaping gas, did not cease at 1 mole, a sign of the fact that under the prevailing test conditions there occurs not only hydrogenolysis of the carbobenzoxy group (XIII→XIV→XV), but also marks the start of intermolecular polyacylation of the resulting bifunctional derivative XV (XV→XVI). In order to further the latter process, and after the yield of CO_2 (totaling 1.36 mols) had practically ceased, the reaction mixture was mildly warmed (60°C) following addition of 1 mole of triethylamine. It is to be assumed that the mixed anhydride XV is able to transfer not only the acyl group of the L-L-start dipeptide (H_2N-A-CO-) to an amino group, but also -- if in smaller proportion -- its carbethoxy group. In the latter case, however, the intermolecular polyacylation stops, since the N-carbethoxy group cannot be hydrogenolyzed. Although this premature cessation of intermolecular polyacylation cannot be avoided, the test results nevertheless show that the polydisperse conversion product XVI also contains components of relatively high molecular weight.

Taking into consideration the observation of Boissannas and Schuman (17), that the formation of ester anhydride from a carboxylic acid anion and chloroformic acid ester is not essentially hampered by the simultaneous presence of free amino groups, the aforementioned procedure could now be simplified, in that the temporary blocking of the amino group of the L-L-start dipeptide

(Ia→XII) for the purpose of production of the mixed esteric hydride XV could be dispensed with. The formation of the polyester XVI also occurs when an ice cold solution of dimethyl-formamide of the L-L-start dipeptide (Ia) is first treated with 1 mole of triethylamine and 1 mole of chloroformic acid-ethylester (Ia-XV) and then, following admixture of a second mole of triethyl-amine, is mildly warmed (XV-XVI). It should be noted that the polyester was obtained in water soluble form, following evaporation of the solvent, and therefore can be dialyzed against water. In order to minimize the possibility of N-carbalkoxylation during the second reaction phase, another test utilized isopropylester (18) instead of chloroformic acid-ethylester; simultaneously and prior to the reaction the dimethyl-formamide solution of the L-L-start dipeptide (Ic) was dehydrated by means of azeotropic distillation with toluene. Here, too, the formation of the polyester (XVII) progressed smoothly; however, the increase in yield was negligible (43.5%, against 38.5% of the theoretical yield). It was found, however, that free poly acid purified by dialysis could be obtained in a better yield from this polyester (see table 2), a sign of a larger fraction of higher molecular components in this polyester.

(2) The second method, utilizing Wieland and Bernhard's (19) peptide and polypeptide synthesis, was applied to all three stereo-isomeric start dipep-tides. Accordingly the carbobenzoxy derivative of the L-L-, D-D- or L-D-start dipeptide (XII from Ia, Ib or Ic) was converted via the mixed anhydride (XIII) with thiophenol to thiophenylester XVIII, and from it the hydrobromide of the start dipeptide-thiophenylester (XIX) was formed by means of glacial acetic acid-hydrogen bromide. When its amino group is liberated in acetone solution by means of triethylamine, then the resulting primary product XX is subjected to intermolecular polyacylation, leading to the polyester XXI, a process that can be furthered by warming (see formula on page 217).

(3) The third method is based on processes first used by Sheehan and Hess (21) in the synthesis of di- and tripeptides. As is known, a dipeptide can be produced by this method in such a way that an amino acid with a protected (for example, acylated) amino group XXII) and an amino-acid ester (XXIII) can be condensed by means of dicyclohexylcarbodiimide (XXIV). The reaction takes place at room temperature, whereby the byproduct dicyclohexylurea (XXVI) results, in addition to the dipeptide derivative XXV. (See formula on page 218).

The mechanics of the condensing effect of dicyclohexylcarbodiimide (and other carbodiimides) was explained by Khorana (22). We are dealing with the activation of the carboxyl group, the first phase of which consists of the following: The reaction components with a free carboxyl group take on carbodiimide, whereby the O-acylated iso-urea derivative (XXVIIa) is formed, which is inherently able to transfer its acyl group to amino groups. It was concluded that this method, which could serve in the development of a single peptide bond, could also be used in poly-autocondensation of the bifunctional start dipeptide (I), provided the free amino group — in spite of its nucleophilic nitrogen atom — does not decisively prevent the formation of the reactive intermediate product (XXVIIb). Such a competitive reaction would lead to the formation of a guanidine derivative (XXVIII), which is probably stable, and thus would bring about a termination of the intermolecular polyacylation process.

Model tests with dicyclohexylcarbodiimide and various primary amines have shown that formation of guanidine derivatives is impossible under the test conditions used in connection with poly-autocondensation of the start dipeptide (I). This is confirmed by tests with the L-L- and D-D-start dipeptide (Ia and Ib): If dicyclohexylcarbodiimide (XXIV) is dissolved in the dimethyl-formamide solution of the start dipeptide (I), the result after a few minutes, even at

room temperature, is the crystalline precipitation of dicyclohexylurea (XXVI) as a sign of poly-autocondensation (see formula on page 218). The filtrate contained the gamma-poly-L-glutamic acid-alpha-methylester (IIa) or the gamma-poly-D-glutamic acid-alpha-methylester (IIb), respectively, which could be transformed into the respective polyacid.

It should be noted here that the carbodiimide method for production of monotonous polypeptides was tested for the first time during synthesis of alpha gamma-poly-glutamic acid (10).

(4) The fourth method was developed from the polypeptide synthesis of Noguchi and Hayakawa (33). According to the original process of the japanese scientists, an oligopeptide ester is first transformed to N-carbothiophenyl derivative by means of chlorothioformic acid-phenylester ($ClCOSC_6H_5$), then the ester group is subjected to acid saponification, and finally the benzene-pyridine solution of N-carbothiophenyl oligopeptide (XXIX) is warmed, whereby the polypeptide (XXX) is formed with separation of carbon dioxyde and thiophenol. As is known, we are dealing here with the activation of the amino group; the process which leads to the polypeptide can be formulated thus: (see formula on page 219).

Formally, a polypeptide is formed here, whose (terminal) amino group occurs in carbothiophenylized form; however, such groups can be readily regenerated to free amino groups even by a mildly alkaline hydrolysis.

Preliminary tests showed that there is also a carboxyl group which can be "activated" with chlorothioformic acid-phenylester in the presence of triethylamine, whereby the first result should be a mixed anhydride of the type XXXI. It was therefore to be expected that Noguchi's and Hayakawa's method could be applied directly to the start dipeptide with chlorothioformic acid phenylester in the presence of triethylamine, there may result, aside from the

N-carbothiophenyl start dipeptide (XXIX; A as in XVIII), the mixed anhydride XXXII, which would be capable of intermolecular polyacylation, just as the N-carbothiophenyl start dipeptide (XXIX). Tests with the L-L-start dipeptide (IIa) showed the following: If a suspension of the L-L-start dipeptide is mixed with a nearly equimolecular amount of chlorothioformic acid-phenylester in ice cold, triethylamine-containing chloroform, and the mixture is shaken, then a considerable portion of the used start dipeptide will immediately dissolve through self-heating of the mixture. From the solution a colorless product ("primary conversion product") is isolated; if the benzene-pyridine solution of this substance is warmed (90°C), then a gradual liberation of a substance of high molecular weight takes place, which, according to its properties and analytical data represents the gamma-poly-L-glutamic acid-alpha-methylester (IIa).

Since the labile "primary conversion product," which probably is non-homogeneous, has not been wholly defined as yet, we have started further examinations, in order to clarify the foregoing reactions, and which shall be discussed later. For the time being it should be noted that the whole procedure is much more complicated. Mixed anhydrides of the type XXXI namely cannot be isolated, since they shift to carboxylic acid-hydrides (XXXIII) immediately following formation. It is assumed that this shift is connected with disproportioning, which progresses in accordance with the equation XXXI ⟶ XXXIII + XXXIV: (see formula on page 220).

It should be possible to immediately intercept the heretofore unknown anhydride XXXIV whose capability for existence seems very questionable, by means of amino groups, whereby, in addition to carbon dioxyde and thiophenol, there should form an N-carbothiophenyl derivative (type XXIX). It has been discussed elsewhere (24), just what conceptions can be arrived at regarding

the composition of the "primary conversion product" and about the mechanism of its conversion to polyester (IIa).

Supplementally to the aforementioned four methods it should be repeated that Waley (14) has effected the synthesis of the L-L-start dipeptide (Ia), independently. He has also accomplished a poly-autocondensation of the L-L-start dipeptide to gamma-poly-L-glutamic acid-alpha-methylester (IIa), and this by means of tetra-ethylpyrophosphite, i.e. with a method first used by Anderson et al (25) in peptide synthesis. It should be noted that the gamma-poly-L-glutamic acid (IIIa) obtained by Waley (14) by saponification of the polyester, contains 0.6% bound phosphorus. We have duplicated the process exactly, and found it less satisfactory than methods 1-4. It should further be noted that Waley to date has not reported on the synthesis of the D-D-start dipeptide (Ib) and the L-D-start dipeptide, nor on their poly-autocondensation.

Comparative degradation of the synthetic gamma-poly-L-glutamic acid-alpha-methylester.

In order to obtain a corresponding comparison with APP and SPP esterified with methanol, gamma-poly-L-glutamic acid-alpha-methylester (IIa), produced by the second method, was converted with liquid ammonia to the corresponding polyamide (IIa; R equals NH_2 instead of R equals OCH_3), then subjected to Hofmann's degradation and immediately hydrolyzed with hydrochloric acid. From the hydrolyzate beta-formyl propionic acid was isolated in the form of its p-nitrophenylhydrazone; whereas alpha gamma-diamino butyric acid, the degradation product (8,9) characteristic of the alpha-glutamyl bond, could not be demonstrated. As is known (7), the identical degradation of the polymethylester produced from APP and SPP resulted in the same conclusion, which has been considered as proof of the predominance of gamma-glutamyl bonds.

Production of gamma-poly-glutamic acids from the corresponding polyesters.

It has been mentioned that, in order to obtain free polyacids, the variously produced polyesters were subjected to alkaline saponification, and the polyacids were isolated via their difficultly soluble copper(II)salt. One must take into account that during these operations final carboxyl and amino groups may be liberated, which should occur in blocked form in the synthetic polyester (for example XVI, XXI, XXX), but a regeneration of amino groups which possibly combined with guanidine groups (type XXVIII) during the course of the third method can hardly be expected.

The stereo-isomeric gamma-poly-glutamic acids obtained in the above manner, i.e. gamma-poly-L-glutamic acid (IIIa), gamma-poly-D-glutamic acid (IIIb) and mesoid gamma-poly-glutamic acid (IIIc), represent snow white products resembling down, which are easily soluble in water; they do not show a biuret reaction, and their ninhydrin reaction is only very weakly positive in comparison to gamma-L-glutamyl-L-glutamic acid (for quantitative data see the test chapter). All of these properties are equally applicable to APP and SPP.

The question presents itself here, whether the alkaline saponification of gamma-poly-glutamic acid-alpha-methylester might not be accompanied by an intramolecular, partial alpha---gamma transpeptidization. Battersby and Robinson (26) namely have found that alkaline saponification of the ethylester of N-acetyl-diglycyl-alpha-L-glutamyl-glycine-hexylamide (partial formula: XXXV) is connected with a far-reaching alpha---gamma transpeptidization (and complete racemization), so that a mixture of structure-isomeric peptide XXXVII and XXXVIII is produced, in which the derivative with a gamma-glutamyl bond even predominates. This process may be explained thus: A 2:6-diketopiperidine derivative (XXXVI) is produced by means of transitional cyclization, which, as

secondary acid amide, undergoes a hydrolytic ring cleavage (XXXVII←XXXVI→XXXVIII) (see formula on page 221). In this connection it should be noted that intramolecular transpeptidization of structure-isomeric glutamyl peptides (type XXXVII and XXXVIII) with a free carboxyl group has been effected in our institute previously (27), although by means of a different method, and this in both directions (alpha⇄gamma). Thereby the intermediate cyclic compounds (type XXXVI) were obtained in crystalline form and their bi-directional hydrolytic ring opening (XXXVII←XXXVI→XXXVIII) (under the effects of diluted lye) was proved beyond a doubt (28).

Since, according to our experience (28), this transpeptidization always produces a mixture in which the gamma-glutamyl compound (XXXVII) exceeds the alpha-glutamyl compound (XXXVIII) by approximately tenfold, it will not be necessary to anticipate an extensive gamma→alpha transpeptidization upon saponification of gamma-poly-glutamic acid-alpha-methylester. Moreover, the gamma→alpha transpeptidization is made more difficult here by the fact that during the approach of the esterified carboxyl group of a gamma-glutamyl residue (XXXVII, $COOCH_3$ instead of COOH) to the respective nitrogen atom, two long chains (R and Q) must co-vibrate. Conversely, one can anticipate that upon alkaline saponification of an alpha-poly-glutamic acid-gamma-ester (partial formula XXXV) the necessary chain vibration will occur more easily and for this reason an alpha→gamma transpeptidization will take place more effortlessly.

In full accord with these conclusions, Volcani (29) has found that an enzyme isolated by him, which specifically splits gamma-L-glutamyl bonds, degrades our synthetic gamma-poly-L-glutamic acid (IIIa) up to 95% to L-glutamic acid. This may be taken as proof of a very low percentage of alpha-glutamyl bonds in this synthetic product, and also as proof that no important

racemization has taken place during the course of the synthesis.

Moreover, the optical purity of some of our end products was tested by complete hydrolysis with hydrochloric acid; the specific rotatory power of the resultant glutamic acid was only slightly lower than that of optically pure L or D-glutamic acid. These tests must be repeated on a larger scale and extended to all end products. It should be mentioned here that gamma-poly-L-glutamic acid (IIIa) obtained by the second method showed the specific rotatory power of $[\alpha]_4^{20}$ equals -23.8° in water, while the specific rotatory power of its natural antipode (IIIb), i.e. of APP, measures $[\alpha]_D^{20}$ equals +23.5°. This excellent agreement could hardly be considered a strict proof of the optical purity of the synthetic product, since the specific rotatory power depends on the molecular weight of the polyacid; an ironclad determination of molecular weight has not yet been accomplished, however (see below).

As in all syntheses of materials of high molecular weight, polydisperse products were formed. It was stressed that preparations of higher mean molecular weight should be removed from the mixtures by means of constant dialysis — unless this decreses the yield — if they can no longer be partitioned paper chromatographically (phenol-water) and remain immovable at the starting point. Dependable determinations of the molecular weight of our products are still lacking; for the time being their average molecular weight has been concluded from their amino nitrogen values (van Slyke), just as this had been done previously with APP and SPP. The amino nitrogen values of our synthetic end products fluctuate (depending on the extent of the dialysis, which had to be curtailed in some cases due to shortage of materials) between 0.16 and 1.5% (in connection with the mesoid polyacid (IIIc) only a preparation with the amino nitrogen value of 2.9% could be obtained so far, due to shortage of materials), while the amino nitrogen values of APP and SPP preparations

obtained by the original isolation method (1) showed a fairly constant 0.18-0.20%. Due to the splitting of the N-terminal gamma-glutamyl bond, which has been observed (30) in connection with van Slyke's method — at least in the case of oligopeptides — the average molecular weights, which are measured by amino nitrogen values, are probably distorted to unreality toward the lower values. The extent of this distortion is shown elsewhere (24). Nevertheless the ratio of their average molecular weight may be approximated from the amino nitrogen values of natural polyglutamic acids obtained by the original method (1),— provided van Slyke's determination is effected on all samples under exactly identical test conditions. This comparative evaluation of relative molecular weight is admissible with the natural products, because they are practically homodisperse. On the other hand, this comparison is hardly valid in dealing with the polydisperse synthetic products (unless it be a comparison of one with the other, or with APP or SPP), especially since the average molecular weight has no real value without knowledge of the quantitative distribution of the individual components. However, if the possibility of presence of cyclopeptides is ignored, then it may be assumed, without committing a gross error, that, for example, a synthetic polydisperse gamma-poly-glutamic acid with an amino nitrogen value of 0.5% (calculated molecular weight 2800) may contain components whose molecular weight reaches that of the natural product (amino nitrogen value 0.2%, calculated molecular weight 7000). In this connection it is to be assumed that the real molecular weight may amount to six times the value which was computed from the amino nitrogen contents by means of van Slyke's method (24).

In the near future we plan to make an ironclad determination of the average molecular weight by means of other methods. At present, tests with

larger amounts of starting material are under way, whose first goal is the production of gamma-poly-glutamic acids of much higher molecular weight and a closer approach to homodispersity.

As long as the tests with larger starting amounts are incomplete, a final decision cannot be made on the most reliable method of synthesis among the four mentioned above, since this evaluation is interdependent on the molecular weight, the yield (computed from the start dipeptide) and the assured means of production. Moreover, the optical purity of all end products must be tested on larger amounts of the substances. Judging by test results to date, which are summarized in Table 2, the first method with utilization of chloroformic acid-i-propylester seems to be the most favorable (yield 9.6% of the theoretical yield, applied to the start dipeptide; amino nitrogen after van Slyke 0.64%).

Serologic comparison of the isomeric gamma-poly-glutamic acids with APP and SPP, respectively.

Prof. Ivanovics (Microbiological Institute of Szeged University) has subjected the synthetic, stereo-isomeric gamma-poly-glutamic acids to serologic tests and has examined polyglutamic acids of other types in the same manner, which also were synthesized in our institute. Professor Ivanovics himself reports on these tests in detail (see appendix). In this connection it should be stressed that solutions of gamma-poly-D-glutamic acid (just as solutions of APP and SPP) diluted up to 10^6 showed a precipitating reaction in antianthrax immune sera, while this reaction did not occur with gamma-poly-L-glutamic acid. Simultaneously, the polydispersity of the synthetic products was proved by means of serologic tests after Ouchterlony (31).

It remained to be determined that all properties of the synthetic gamma-poly-D-glutamic acid correspond to those of anthrax polypeptide. On these

grounds and the results of previous degradation tests (7) it may now be affirmed that the anthrax polypeptide is to be considered as being gamma-poly-D-glutamic acid (IIIb). By the synthesis of this polyglutamic acid the total synthesis of a natural hapten has been accomplished for the first time.

In regard to the constitution of the subtilis polypeptide it must still be decided whether we are dealing here with a mixture of gamma-poly-D-glutamic acid (IIIb) and gamma-poly-L-glutamic acid (IIIa), or whether glutamic acid components of opposed configuration may occur in association in one polypeptide chain. According to observations made by Prof. Ivanovics the mesoid gamma-poly-glutamic acid (IIIc) also showed a positive serologic reaction. Consequently, the possibility of associated occurrence of D and L-glutamic acid components in the peptide chains of SPP cannot be ruled out a priori.

Finally it should be stressed that only gamma-poly-D-glutamic acid and mesoid gamma-poly-glutamic acid showed a positive serologic reaction, while heretofore tested poly-glutamic acids of different constitution have proved to be inactive serologically.

Description of tests.

Synthesis of the start dipeptide (I) and its carboxyl-activated derivatives.

(a) Synthesis of the L-L-start dipeptide (Ia) and its derivatives.

L-L-start dipeptide; gamma-L-glutamyl-L-glutamic acid-alpha alpha'-dimethylester (Ia). The synthesis of these compounds has already been described by us (12), and independently by Waley (14). Differing observations were pointed out in the theoretical part.

Carbobenzoxy-gamma-L-glutamyl-L-glutamic acid-alpha alpha'-dimethylester (XII; L-L-form). See our previous paper (12). Melting point 109-110°C. $[\text{alpha}]_D^{20}$ equals -28.3 (c equals 11.8; methanol).

Carbobenzoxy-gamma-L-glutamyl-gamma'-L-glutamylthiophenol-alpha alpha'-dimethylester (XVIII; L-L-form). An ice cooled and stirred solution of 3.49 g of carbobenzoxy-L-L-start dipeptide (XII) in 20 ml of absolute tetrahydrofurane was first mixed with 1.12 ml of absolute triethylamine, then, within 15 minutes, a mixture of 0.77 ml of chloroformic acid ethylester and 2 ml of absolute tetrahydrofurance was added drop by drop. After an additional 15 minutes a solution of 0.87 ml of thiophenol in 3 ml of absolute tetrahydrofurane was added drop by drop (within 30 minutes) to the mixture, which was permeated with crystals of separated triethyl ammonium chloride; the mixture was stirred for another hour at room temperature and then allowed to stand for 14 hours. Now the mixture was dried under reduced pressure (bath at 25°C), the residue then washed on the filter, first with a total of 140 ml of water, then, following suction drying, with 20 ml of petrolether, and finally stored overnight in the vacuum exsiccator over P_2O_5 and paraffin. This crude product (3.6 g) was initially recrystallized from 60 ml of a mixture of equal parts of benzol and petrolether (melting point 124-126°C). Following one-time recrystallization of this product, 2.73 g (64.6% of the theoretical yield) of an analytically pure substance with a melting point of 134-135°C was obtained. $[\alpha]_{20}^{D}$ equals -9.6° (c equals 4.0; tetrahydrofurane).

$C_{26}H_{30}O_8N_2S$ (530.6)

Reported: C 58.85, H 5.7, N 5.3, CH_3O 11.7

Found: C 59.0, H 5.7, N 5.7, CH_3O 11.9

Hydrobromide of the gamma-L-glutamyl-gamma'-L-glutamyl-thiophenol-alpha alpha'-dimethylester (XIX; L-L-form). 3.5 g of the pure L-L-thiophenylester XVIII was dissolved in 35 ml of absolute glacial acetic acid-hydrogen bromide (10% HBr), the solution was allowed to stand for 1 hour, then mixed by stirring

with 100 ml of absolute ether, whereby the hydrobromide XIX separated as a sticky product. Following pouring off the fluid, the product was treated three times with 100 ml of absolute ether each, causing it to turn into a powder. This was washed twice more with 50 ml of absolute ether each (suspension, pouring off), then dried in the vacuum exsiccator over sulfuric acid (3.0 g). The product is very hygroscopic. Attempts at crystallization were unsuccessful.

(b) Synthesis of the D-D-start dipeptide (Ib) and its derivatives.

Carbobenzoxy-D-glutamic acid-gamma-hydrazide (IV; D-Form). The compound was produced in the same manner as the corresponding derivative of L-glutamic acid (12). First the hydrochloride of D-glutamic acid-gamma-methylester (30 g; melting point 156-158°C; $[\alpha]_D^{20}$ equals -24.2°(c equals 9.35; water) was obtained from D-glutamic acid-hydrochloride (52 g), then 28 g of the ester-hydrochloride yielded carbobenzoxy-D-glutamic acid-gamma-methylester, which was mixed with hydrazinhydrate while still in its crude form. The initially resulting , crystalline carbobenzoxy-D-glutamic acid-gamma-hydrazide (27 g; 64% of the theoretical yield) melted at 168-169°C (decomposition) and was suited for further treatment. After one-time recrystallization from aqueous alcohol (1:2) an analytically pure product was obtained which melted at 177-178°C. $[\alpha]_D^{20}$ equals +11.5° (c equals 10.75; n-hydrochloric acid).

$C_{13}H_{17}O_5N_3$ (295.3)

Reported: C 52.9, H 5.8, N 14.2

Found: C 52.3, H 5.85, N 14.1

D-glutamic acid-gamma-benzylester (VI; D-Form). By use of the method described in the production of L-stereo-isomers (12), 22 g of D-glutamic acid yielded 13.8 g (39% of the theoretical yield) of D-glutamic acid-gamma-benzyl-

ester with a melting point of 167-168°C, which had been recrystallized once from water and which was suited for further treatment. The melting point remained constant after several recrystallizations. $[\alpha]_D^{20}$ equals -28.1° (c equals 9.8; n-hydrochloric acid).

$C_{12} H_{15} O_4 N$ (237.25)

Reported: C 60.8, H 6.3, N 5.9

Found: C 60.65, H 6.2, N 6.1

Carbobenzoxy-gamma-D-glutamyl-D-glutamic acid-gamma'-benzylester (VII; D-D-Form). Produced in an analogous manner to the stereo-isomeric L-L-product (12) from 23.7 g of carbobenzoxy-D-glutamic acid-gamma-hydrazide (IV) with a melting point of 168-169°C and 18.9 g of D-glutamic acid-gamma-benzylester (VI). The yield of directly resulting crystalline product was 21.4 g (61% of the theoretical yield, upon deduction of the unchanged benzylester, 2.2 g). Melting point: Drawn 153-159°C, after sintering 138°. Although the product is rendered impure by the structure-isomeric compound IX (see the general part and elsewhere (12), further treatment was nevertheless continued. For the purpose of analysis a sample (2 g) was recrystallized once from alcohol (10 ml). Melting point 160-162°C, after sintering 156°. $[\alpha]_D^{20}$ equals +6.7° (c equals 4.7; methanol).

$C_{25} H_{28} O_9 N_2$ (500.5)

Reported: C 60.0, H 5.6, N 5.6

Found: C 59.6, H 5.7, N 5.6

Carbobenzoxy-gamma-D-glutamyl-D-glutamic acid-alpha alpha'-dimethyl-gamma'-benzylester (VIII; D-D-Form). 7 g of carbobenzoxy-gamma-D-glutamyl-D-glutamic acid-gamma-benzylester (VII) with a melting point of 153-159°C (see

above) yielded 5 g (67.7% of the theoretical yield) of substance VIII with a melting point of 116-117°C when subjected to the method used in the production of the L-L-form (12); it was treated further. A small sample was recrystallized once from methanol for analysis. Melting point 119-120°C. $[\alpha]_D^{20}$ equals +7.3° (c equals 4.9; acetic ester).

$C_{27}H_{32}O_9N_2$ (528.5)

Reported: C 61.35, H 6.1, CH_3O 11.7

Found: C 61.7, H 6.1, CH_3O 11.8

Previous data (12) on the specific rotatory value of the L-L-antipode must be revised on the basis of new control determinations to $[\alpha]_D^{20}$ equals -7.1° (c equals 5.5; acetic ester).

D-D-start dipeptide; gamma-D-glutamyl-D-glutamic acid-alpha alpha'-dimethylester (Ib). 8 g of the above product (VIII) with a melting point of 116-117°C yielded 3.1 g (64% of the theoretical yield) of the D-D-start dipeptide (Ib) by means of the method described in connection with the production of the L-L-start dipeptide (Ia) (12). The directly resulting crystalline product, washed first with methanol-ether, then with ether, melted upon sintering and rapid heating at 129-130°C. The product contains 1 mole of crystal water. $[\alpha]_D^{20}$ equals +3.3° (c equals 4.86; methanol). Previous data (12) on the specific rotatory value of the L-L-antipode must be revised on the basis of new control determinations to $[\alpha]_D^{20}$ equals -3.2° (c equals 4.72; methanol).

$C_{12}H_{20}O_7N_2 \cdot H_2O$ (322.3)

Reported: C 44.7, H 6.9, N 8.7, CH_3O 19.2

Found: C 45.2, H 6.75, N 8.6, CH_3O 19.2

Examination of the uniformity of compounds VII and VIII (D-L-form), and

of the D-D-start dipeptide (Ib). 0.3 g each of product VII (D-D-form) with a melting point of 160-162°C and its methylated derivative (VIII; D-D-form) with a melting point of 119-120°C were hydrogenolysed (Pd-animal charcoal, 10% Pd) in 80% acetic acid (25 ml). The filtrate was boiled down under reduced pressure and the two residues, along with 0.3 g of the D-D-start dipeptide (Ib) with a melting point of 129-130°C were dissolved in 50 ml of water each. 0.01 ml of each of these solutions was chromatographed. Solvent: Acetic acid-n-butanol-water 1:4:5; ascending chromatography on Macherey-Nagel paper #518; development with ninhydrin. Substance VII yielded two slightly fused spots, distinguishable by their differing intensity, R_f equals 0.21-0.24, which points to the presence of gamma-D-glutamyl-D-glutamic acid and alpha-D-glutamyl-D-glutamic acid. Substance VIII showed a very intensely colored spot of the gamma-D-glutamyl-D-glutamic acid-alpha alpha'-dimethylester (R_f equals 0.50), and a much smaller and only very weakly colored spot of the alpha-D-glutamyl-D-glutamic acid-gamma alpha'-dimethylester (R_f equals 0.34). In connection with substance Ib the spot R_f equals 0.50 is very intensely colored, while the considerably smaller spot R_f equals 0.34 barely reached the border of sensitivity (about 0.2 gamma) of the ninhydrin reaction of the alpha-D-glutamyl-D-glutamic acid-gamma alpha'-dimethylester (XI). Consequently this impurity in the D-D-start dipeptide (Ib) with a melting point of 129-130°C amounts to less than 0.5%.

Carbobenzoxy-gamma-D-glutamyl-D-glutamic acid-alpha alpha'-dimethylester (XII; D-D-form). By utilization of the method used in carboxylation of the L-L-start dipeptide, 2.65 g of the D-D-start dipeptide (Ib) yielded 1.68 g (47% of the theoretical yield) of its carbobenzoxy derivative (XII) in an analytically pure state. Melting point 109-110°C. $[\alpha]_D^{20}$ equals +28.3 (c equals 9.8; methanol).

Carbobenzoxy-gamma-D-glutamyl-gamma'-D-glutamyl-thiophenol-alpha alpha'-dimethylester (XVIII; D-D-form). With the method used for production of the stereo-isomeric L-L-compound (XVIII; L-L-form), 1.54 g of the carbobenzoxy-D-D-start dipeptide produced 1.15 g (61.7% of the theoretical yield) of the D-D-compound (XVIII) in an analytically pure state. Melting point 133-135°C.

Hydrobromide of the gamma-D-glutamyl-gamma'-D-glutamyl-thiophenol-alpha alpha'-dimethylester (XIX; D-D-form). From 1.1 g of the D-D-thiophenylester XVIII, utilizing the production method of the hydrobromide XIX (L-L-form), the crude D-D-hydrobromide (XIX) was obtained in form of a hygroscopic powder of nearly quantitative yield.

(c) Synthesis of the L-D-start dipeptide and its derivatives.

All steps were in imitation of the synthesis of the L-L-start dipeptide and its derivatives. In the following, only the amounts of material, yield, method of crystallization, analysis data and physical constants are given.

Carbobenzoxy-gamma-L-glutamyl-D-glutamic acid-gamma'-benzylester (VII; L-D-form). From 11.8 g IV (L-form) and 7.7 g VI (D-form) 8 g (47% of the theoretical yield) of the crude product. Once recrystallized from 180 ml of acetic ester-ether (1:1) 5.24 g, from the mother liquor an additional 2.2 g melting point 135-137°C. Once more recrystallized from acetic ester: Melting point 137-138°C. $[\text{alpha}]_D^{20}$ equals -1.12° (c equals 8.9; methanol).

$C_{25}H_{28}O_9N_2$ (500.5)

Reported: C 60.0, H 5.6, N 5.6

Found: C 60.1, H 5.5, N 5.3

Carbobenzoxy-gamma-L-glutamyl-D-glutamic acid-alpha alpha'-dimethyl-gamma'-benzylester (VIII; L-D-form). From 7.4 g VII (L-D-form) 4.1 g (52% of the theoretical yield) VIII (L-D-form) with a melting point of 104-107°C. Re-

crystallized from absolute acetic ester (12 ml) 3.15 g with a melting point of 107-108.5°C. $[\alpha]_D^{20}$ equals +0.95° (c equals 11.5; acetone).

$C_{27}H_{32}O_9N_2$ (528.5)

Reported: C 61.35, H 6.1, N 5.3, CH_3O 11.7

Found: C 61.6, H 6.3, N 5.3, CH_3O 12.0

L-D-start dipeptide; gamma-L-glutamyl-D-glutamic acid-alpha alpha'-dimethylester (Ic). From 3.1 g VIII (L-D-form) 1.2 g (66% of the theoretical yield) of L-D-start dipeptide once recrystallized from methanol (7 ml). Melting point 143-144°C. $[\alpha]_D^{20}$ equals +38.6° (c equals 5.5; methanol).

$C_{12}H_{20}O_7N_2$ (304.3)

Reported: N 9.2, CH_3O 20.4

Found: N 9.4, CH_3O 20.3

The chromatographic examination of the product's uniformity was conducted as in the case of the D-D-start dipeptide and furnished the same result.

Carbobenzoxy-gamma-L-glutamyl-D-glutamic acid-alpha alpha'-dimethylester (XII; L-D-form). From 1 g L-D-start dipeptide (Ic) 0.5 g (35% of the theoretical yield) of the crystalline crude product; from 4 ml of absolute acetic ester-ether (1:1) twice recrystallized 0.35 g with a melting point of 136-137°C. $[\alpha]_D^{20}$ equals +4.3° (c equals 8.2; methanol).

$C_{20}H_{26}O_9N_2$ (438.4)

Reported: C 54.8, H 6.0, N 6.4

Found: C 55.0, H 6.1, N 6.7

Carbobenzoxy-gamma-L-glutamyl-gamma'-D-glutamyl-thiophenol-alpha alpha'-dimethylester (XVIII; L-D-form). First, an oil permeated with crystals

resulted from 3 g of the carbobenzoxy-L-D-start dipeptide (XII; L-D-form) subjected to the process used in the production of the stereo-isomeric L-L-compound (XVIII; L-L-form). This oil was dissolved in acetic ester, and the solution first shaken in water in order to remove the triethyl ammonium chloride, then dried (Na_2SO_4) and finally condensed under reduced pressure. The resultant oily residue crystallized slowly upon trituriation with absolute ether. The crystalline product was first recrystallized once from absolute methanol-ether (1:4), then once from absolute methanol. The product (0.65 g; 17.9 of the theoretical yield) melted spottily at 97-100°C, after sintering at 94°. Due to shortage of materials further purification was omitted.

Hydrobromide of the gamma-L-glutamyl-gamma'-D-glutamyl-thiophenol-alpha alpha'-dimethylester (XIX; L-D-form). By use of the method for production of hydrobromide XIX (L-L-form), 0.62 g of L-D-thiophenylester XVIII yielded crude L-D-hydrobromide (XIX) in the form of a hygroscopic powder in a nearly quantitative yield.

Conversion of the stereo-isomeric start dipeptide (I) and its derivatives to gamma-poly-glutamic acid-alpha-methylester of the respective configuration.

First method. (a) Production of L-polyester (IIa or XVI) from the carbobenzoxy-L-L-start dipeptide (XII; L-L-form). In a double-necked flask a solution of 1.46 g of carbobenzoxy-gamma-L-glutamyl-L-glutamic acid-alpha alpha'-dimethylester (XII; L-L-form) in 3 ml of the purest, waterless dimethyl formamide, under exclusion of moisture, by ice cooling and stirring, was mixed first with 0.47 ml of absolute triethylamine, then drop by drop with a mixture of 0.32 ml chloroformic acid ethylester and 2 ml dimethyl formamide. Stirring was continued for 10 more minutes. Then the solution which was permeated with the crystals of separated triethyl ammonium chloride, was mixed with 0.5 g of Pd-animal charcoal (10% Pd); one neck was stopped with a gas

lead-in tube reaching the bottom of the flask, the other was connected with a wash bottle filled with concentrated sulfuric acid and equipped with Geissler's potash apparatus of known weight. Now a slow current of hydrogen (1 bubble per second) was allowed to pass through the mixture under constant ice cooling. It could be determined by temporary interruption of the hydrogen supply and weighing of the potash apparatus that within 5 hours about 1 mole (0.15 g) of CO_2 had escaped. Since the yield of CO_2 had not ceased at this stage, the treatment was continued. After an additional $2\frac{1}{2}$ hours the escape of CO_2 was practically completed and amounted to a total of 1.36 mole (0.20 g). Now the mixture was centrifuged, the sediment washed once by soaking in 3 ml dimethyl formamide and subsequent centrifugation. The unified solution, after addition of 0.47 ml of triethylamine, was allowed to stand for 3 hours at room temperature, then warmed to 60°C for one hour and finally boiled down (bath 80°C) in a vacuum (20 mm), whereby a viscous brown oil was obtained. This was thoroughly trituriated 8 times in 40 ml of absolute ether each, then stored in a vacuum exsiccator over sulfuric acid. The resultant product, which was greyish due to the adhering catalyst, weighed 177 mg (18.6% of the theoretical yield when applied to the carbobenzoxy-L-L-start dipeptide, about 9.3% if applied to the L-L-start dipeptide). The crude product (polyester 1-a) was treated further without purification.

(b) Production of the L-polyester (IIa) directly from the L-L-start dipeptide (Ia). By means of chloroformic acid ethylester. A solution of 2.0 g of the L-L-start dipeptide (Ia) in 11 ml of dimethyl formamide was first mixed by stirring and cooling (-4°C) with 0.87 ml of triethylamine, then drop by drop with a mixture of 0.53 ml of chloroformic acid ethylester and 3 ml of dimethyl formamide. Stirring was continued for another 15 minutes, then the precipitated triethyl ammonium chloride was quickly filtered by suction and washed twice

with 2 ml of dimethyl formamide each time. The wash fluid was united with the filtrate and compressed to about 4 ml in a high vacuum (bath 0°C). This solution was mixed with 0.87 ml of triethylamine and warmed to 40-60°C for 8 hours, then allowed to stand overnight, whereby a slight crystalline separation took place. Ignoring the latter, the mixture was boiled down completely in a vacuum (bath 80°C), the residue was dissolved in 5 ml of methanol, evaporated once more under reduced pressure and the last process was repeated. The resulting reddish-brown, viscous oil (2.7 g) was dissolved in 15 ml of water, the solution was dialysed (cellophane) for 46 hours against a total of 2 l of distilled water (in four parts), then subjected to freeze drying and the solid polyester stored for 24 hours in the vacuum exsiccator over P_2O_5. The sand-colored, amorphous product (polyester 1-b) weighed a total of 688 mg (38.8% of the theoretical yield); it was treated further without purification.

$[C_6 H_9 O_3 N]_n$ $[143.14]_n$

Reported: CH_3O 21.7

Found: CH_3O 22.0

By means of chloroformic acid-i-propylester. A solution of 2.0 g of the L-L-start dipeptide in 12 ml of absolute dimethyl formamide was mixed with 3 ml of absolute toluol and compressed to about 6 ml in a vacuum (8 mm; bath 50°C), then the partly precipitated substance was dissolved again by addition of 6 ml of dimethyl formamide and mild warming. The solution was mixed first with 0.87 ml of triethylamine, then, accompanied by strong cooling (bath -10°C), stirred drop by drop with a mixture of 0.72 ml of chloroformic acid-i-propylester and 2 ml of dimethyl formamide. Stirring and cooling continued for another 20 minutes, then triethylamine (0.87 ml) was added and gradually brought to room temperature. Now the solution, permeated with crystals (triethyl ammonium chloride) was warmed to 50°C for 3 hours, then to 70°C for one

hour and then boiled down in a vacuum (10 mm). The residue was trituriated with 30 ml of absolute ether and the mixture stored overnight in the refrigerator. After pouring off the ether, the viscous product, permeated with crystals, was absorbed in 20 ml of methanol, the mixture was vigorously boiled down in a vacuum, and this process repeated once more. The residue was dissolved in 25 ml of distilled water, the solution first dialysed (cellophane) against 3½ l. of distilled water (in 4 parts) for 72 hours, then frozen dry. The sand-colored, amorphous product (polyester 1-b') weighed 773 mg (43.5% of the theoretical yield).

$[C_6 H_9 O_3 N]_n$ $[143.14]_n$

Reported: CH_3O 21.7

Found: CH_3O 21.0

Second method. gamma-L-glutamic acid-alpha-methylester (IIa, or XXI). To a solution of 3 g of crude hydrobromide XIX (L-L-form) in 3 ml of absolute acetone 1.8 ml of absolute triethylamine was added under exclusion of moisture and the solution allowed to stand for 24 hours. Ignoring the crystalline separation (triethyl ammonium bromide), the major part of the fluid was distilled off by means of a heat bath (100°C), then the oily residue was warmed for 3 hours at 100°C under exclusion of moisture. After cooling, the viscous residue was covered with 150 ml of absolute ether and stored in the refrigerator for 12 hours. This caused the product to harden to such a degree that it could be trituriated (under ether) to a fine powder. This was centrifuged, then washed in the centrifuge 3 times with 70 ml of ether each, 3 times with 30 ml of water each, 3 times with 50 ml of acetone each, and finally twice with 50 ml of absolute ether each. The nearly colorless product thus obtained (L-polyester 2) was dried first by air, then in the vacuum exsiccator over P_2O_5.

Yield 400 mg (21.2% of the theoretical yield applied to carbobenzoxylated thiophenylester XVIII, about 6.8% applied to the L-L-start dipeptide).

$\left[C_6 H_9 O_3 N\right]_n \cdot \left[143.14\right]_n$

Reported: C 50.4, H 6.3, N 9.8, CH_3O 21.7

Found: C 49.6, H 5.7, N 9.6, CH_3O 21.9

gamma-poly-D-glutamic acid-alpha-methylester (IIb, or XXI). The crude hydrobromide XIX (D-D-form) obtained from 1.1 g of carbobenzoxy-gamma-D-glutamyl-gamma'-D-glutamylthiophenol-alpha alpha'-dimethylester (XVIII; D-D-form) was converted by means of the aforementioned method. The product thus obtained (D-polyester 2) weighed 139 mg (23.4% of the theoretical yield applied to carbobenzoxylated thiophenylester XVIII, about 7.2% applied to the D-D-start dipeptide).

mesoid gamma-poly-glutamic acid-alpha-methylester (IIc, or XXI). The crude hydrobromide XIX (L-D-form) obtained from 620 mg of carbobenzoxy-gamma-L-glutamyl-gamma'-D-glutamylthiophenol-alpha alpha'-dimethylester (XVIII) was converted in the aforementioned manner. The product thus obtained (L-D-polyester 2) weighed 220 mg (65.8% of the theoretical yield applied to carbobenzoxylated thiophenylester XVIII, 4.1% applied to the L-D-start dipeptide).

Third method. gamma-poly-L-glutamic acid-alpha-methylester (IIa). 2.3 g (0.11 mole) of freshly distilled dicyclohexylcarbodiimide was dissolved in a solution of 3.2 g (0.1 mole) of the L-L-start dipeptide (Ia) in 6.5 ml of dimethyl formamide. A strong self-heating followed, and about 10 minutes later separation of dicyclohexylurea commenced. The reaction mixture was heated under exclusion of moisture for 4 hours in a boiling water bath, then cooled off. The dicyclohexylurea (1.7 g) was filtered off by suction and washed on

the filter with ice cold dimethyl formamide. The united filtrate was evaporated under reduced pressure (20 mm; bath 90°C), the viscous residue was digested repeatedly with a total of 200 ml of absolute ether until it crumbled into a powder. The product thus obtained (2.4 g) represents a mixture of the amorphous polyester (L-polyester 3) and crystalline dicyclohexylurea (retained amount calculated at 0.8 g). Yield of polyester about 1.6 g (56.3% of the theoretical yield). Since the urea derivative could not be completely removed, the mixture was directly utilized for production of gamma-poly-L-glutamic acid.

gamma-poly-D-glutamic acid-alpha-methylester (IIb). 1.35 g of the D-D-start dipeptide (Ib) yielded, by the aforementioned method, 1.3 g of a powdery product, which represents a mixture of the amorphous polyester (D-poluester 3) and crystalline dicyclohexylurea (about 0.5 g). Yield of polyester about 0.8 g (66.7% of the theoretical yeild). The product was utilized directly for the production of gamma-poly-D-glutamic acid.

Fourth method. gamma-poly-L-glutamic acid-alpha-methylester (IIa). An ice cooled suspension of 3.22 g (0.01 mole) of the L-L-start dipeptide (Ia) in 15 ml of absolute chloroform was first mixed with 1.39 ml (0.01 mole) of triethylamine, then drop by drop by shaking with 1.45 ml (0.01 mole) of chloroformic acid-thiophenylester. A considerable part of the converted substance dissolved immediately by self-heating. The mixture was shaken by machine for 4 hours in a closed container, the unreacted start dipeptide (0.55 g) filtered out, the filtrate shaken twice in water, once in diluted hydrochloric acid, twice more in water, then dried with Na_2SO_4 and finally boiled down at room temperature under reduced pressure. There remained a yellowish, viscous oil which was turned into powder by repeated trituriation with ether. This "primary conversion product," weighing 1.3 g, could not be crystallized. It was

converted directly to the polyester.

The "primary conversion product" (1.3 g) was dissolved in a mixture of 4 ml of absolute pyridine and 16 ml of absolute benzol. Within a few minutes a slight turbidness was noted. The mixture was heated in a bath of 90°C under exclusion of moisture, whereby the separation of the substance progressed and the major portion of the product adhered to the walls of the flask as a film; the solution turned a dirty yellow. After 60 hours the separation of the product was practically completed. The substance was isolated in the centrifuge, washed several times with absolute acetone and then with absolute ether, and finally dried in the vacuum exsiccator over sulfuric acid. The polyester thus obtained (L-polyester 4) represents a lightly sand-colored, amorphous powder. Yield 610 mg (25.7% of the theoretical yield applied to the L-L-start dipeptide, less the part that was not converted). For the purpose of analysis, a sample was dissolved in methanol, precipitated with ether, centrifuged, and dried in the vacuum pistol over P_2O_5 first at room temperature, then at 78°C.

$\left[C_6 H_9 O_3 N\right]_n \quad \left[143.14\right]_n$

Reported: C 50.4, H 6.3, N 9.8, CH_3O 21.7

Found: C 49.5, H 6.1, N 9.9, CH_3O 21.0

Degradation of gamma-poly-L-glutamic acid-alpha-methylester for the purpose of constitutional determination. 100 mg of the polyester obtained by the second method (L-polyester 2) in 10 ml of liquid ammonia was allowed to stand in a bomb tube for 72 hours at room temperature; during this time the substance dissolved almost completely. After the ammonia had escaped from the opened tube, the solid residue was washed in the centrifugal tube twice with 50 ml of water each, twice with a homogenous mixture of alcohol and ether, and finally three times with 30 ml of absolute ether each. The white, amorphous polyamide (IIa;

R equals NH_2 instead of OCH_3), dried first by air, then in the vacuum exsiccator over P_2O_5, weighed 62 mg (69.3% of the theoretical yield). A small sample for analysis was dried for 5 hours more in the pistol over P_2O_5 at 80°C.

$[C_5 H_8 O_2 N_2]_n$ $[128.14]_n$

Reported: N 21.8

Found: N 20.6

It should be mentioned that the polyamide produced in the same manner from SPP via the methylester contained the same amount of nitrogen (20.0%)(32).

50 mg of the polyamide was treated with 0.98 ml of 2.68% alkaline sodium hypochlorite with frequent rotation, first for one hour at room temperature, then 15 minutes at 60°C, whereby the substance dissolved almost completely. After addition of 10 ml of 6n-hydrochloric acid the solution was boiled with reflux for 30 minutes and then evaporated under reduced pressure. The residue was dissolved twice in 2 ml of water each, and the solution boiled down under reduced pressure. The residue thus obtained was mixed with 1 ml of a freshly produced solution of p-nitrophenylhydrazine in n-hydrochloric acid, which had been saturated at room temperature. The mixture was dipped for a few seconds into a bath of 70°C and allowed to stand for 15 minutes under ice cooling. The beta-formylpropionic acid-p-nitrophenylhydrazine, which had separated in crystalline form, was filtered off by suction, washed on the filter twice with 1 ml of ice cold water each and finally dried in the vacuum exsiccator. It weighed 20 mg and melted at 172°C. A single recrystallization from 1 ml of water produced an analytically pure product, which melted at 177°C in agreement with the literature (33) and did not show a depression in a mixture test with an authentic preparation with a melting point of 177°C.

30 mg of the polyamide was treated with sodium hypochlorite in the aforementioned manner, and the solution diluted to 4 ml with water. One half of this

solution was used directly, the other half (control test) after addition of 0.7 mg of alpha gamma-diaminobutyric acid for proof of alpha gamma-diaminobutyric acid, which is characteristic (8,10) of the alpha-glutamyl bond. Each solution was mixed with 2 ml of fuming hydrochloric acid and boiled with reflux for 2 hours. The cooled off solution was extended in the apparatus with ether for the purpose of removal of beta-formylpropionic acid, then steamed dry under reduced pressure. The residue was mixed with 5 ml of ice cold, fuming hydrochloric acid, the separated NaCl filtered off and the filtrate evaporated under reduced pressure. This treatment of the residue with fuming hydrochloric acid was continued until separation of NaCl ceased. The final residue of both samples was dissolved in 1 ml of water each and 0.01 ml from each of the solutions subjected to paper chromatography. (Ascending chromatography on Wh. paper #4; solvent n-butanol-glacial acetic acid-water 4:1:5; development with ninhydrin). While the control test clearly shows the spot of alpha gamma-diaminobutyric acid in addition to the spot of glutamic acid, the original sample failed to produce the first mentioned spot.

Production of gamma-poly-glutamic acids (III) of different configuration from the corresponding polyesters.

Starting quantities of 100-1600 mg of the various polyesters were heated with a 50-100% excess of 0.25n-NaOH for one hour in the steam bath, then the solution was filtered, if necessary (for example, in treating the polyester obtained by the fourth method for the purpose of removing the undissolved dicyclohexylurea), then the copper salt of the polyacid was precipitated at pH 6 (adjusted with hydrochloric acid or soda lye) with a saturated solution of copper(II)sulfate. The copper salt was centrifuged and washed 3-4 times in the centrifuge with a small amount of ice cold water. The moist precipitate was dissolved in the just sufficient amount of 0.1 n-HCl; as a rule this

amounted to 1 ml per 10-15 mg of saturated polyester. This solution was saturated with hydrogen sulfide, the excess of the latter removed with an air current, the precipitate was centrifuged and washed twice in the centrifuge with a small amount of water. The solution, united with the wash water, was clarified by filtration, if necessary, then dialyzed against distilled water by means of a cellophane membrane (dialyzing hose). Dialysis was carried out until the chloride reaction disappeared; the total quantity of exterior water, which had been changed 8-12 times, as a rule amounted to 12-15 ml to each mg of started polyester. The remaining solution was compressed to 10-20 ml in a vacuum (bath 30°C), then subjected to freeze drying. Copper(II)sulfide which might have been carried over in colloidal form coagulates during freeze drying and may be removed completely by dissolving the residue in water, filtration and re-freezing.

The poly-glutamic acids thus obtained represent snow-white, downy products, which are very easily soluble in water. They do not show a biuret reaction -- in agreement with APP and SPP --, their ninhydrin reaction is only weakly positive -- in comparison with gamma-L-glutamyl-L-glutamic acid; while the limit of sensitivity in the reaction (tested on paper) lies at about 0.2 gamma with the latter substance, the synthetic gamma-poly-glutamic acids show a limit of about 10 gamma, in some cases as high as 20 gamma. SPP obtained by the original method (1) has a sensitivity limit of at least 25 gamma. Table 1 shows a few test data; it should be noted that the yields do not apply to the started polyester but the corresponding start dipeptide, and this also in those cases where polycondensation was carried out with the derivative and not the start dipeptide.

Table 1

Prepared product	Started polyester Method	Amount (mg)	Obtained gamma-poly-glu. Amount (mg)	H_2N-N (%)	Yield (%)
(1) gamma-poly-L-glu.	L-polyester 1-a	176	48		1.1
(2) gamma-poly-L-glu.	L-polyester 1-b	688	89	1.18	7.1
(3) gamma-poly-L-glu.	L-polyester 1-b'	762	138	0.61	9.8
(4) gamma-poly-L-glu.	L-polyester 2	100	44	1.50	3.9
(5) gamma-poly-L-glu.	L-polyester 3	~1600	142	0.49	6.3
(6) gamma-poly-L-glu.	L-polyester 4	252	73	0.32	7.8
(7) gamma-poly-D-glu.	D-polyester 2	138	86	1.50	5.4
				(0.82)*	(4.4)*
(8) gamma-poly-D-glu.	D-polyester 3	~900	60	0.16	6.3
(9) mesoid gamma-poly glu.	L-D-polyester 2	220	70	3.6	1.3
				(2.9)**	

* product (7) repeatedly dialyzed, ** product (9) repeatedly dialyzed

All gamma-poly-glutamic acids listed in table 1 show a considerable polydispersity. This was demonstrable not only with the serologically active poly acids by means of Ouchterlony's test method (31) (see the appended report by Ivanovics), but also in all products by chromatographic examination. In ascending chromatography (solvent phenol-water 7:3, development with ninhydrin) we obtained strips emanating from the application point and drawn out in the direction of travel. By further fractional dialysis of these products fractions were dialyzed out whose chromatograms at constant running time (20 hours) showed shorter and shorter strips (emanating from the point of application) until finally the chromatogram of the undialyzed residue consisted of one

single, sharply delineated spot at the point of application.

Water and ash constituents are quite readily bound by synthetic gamma-poly-glutamic acids, as they are by APP and SPP. This has two consequences: Firstly, entirely ashless preparations can hardly be obtained by purification by means of ordinary dialysis, and secondly, the bound water can only be removed sufficiently fast by drying in the high vacuum pistol at 110-120°C; however, at this temperature there already occurs a weak decomposition, as evidenced by slight coloring of the snow white product. The ash contents of our preparations fluctuated between < 0.01 and 1.8%. Due to the strong tendency to bind water a strict control of the end product by means of elementary analysis is not possible; it was determined, however, that the products resulting from freeze drying contain (after storage for 24 hours in the vacuum exsiccator over P_2O_5) almost exactly 1 mole of bound water to every two glutamic acid residues. Table 2 summarizes a few data of the elementary analysis; the drying method is shown in the last column.

It should be noted here that Waley (14) furnishes analytic data of his synthetic gamma-poly-L-glutamic acid, also obtained by freeze drying, (C 40.9, H 5.9, N 9.55), which correspond to 1 mole of bound water to every glutamic acid residue (reported: C 40.8, H 6.2, N 9.5). We did not observe such a combination with SPP or APP, nor with our synthetic products; however, this was found to be the case in synthetic alpha gamma-poly-glutamic acid (10). It should be mentioned further that the low content of methoxyl in some preparations, which points to incomplete saponification of the polyester, does not constitute an insurmountable error in the synthesis. Due to the small starting quantities with which we had to cope we utilized the mildest saponification method possible, in order to prevent possible splitting of the peptide chain.

Table 2

Product	Production method (table 1)	C %	H %	N %	CH_3O %	Ash (sulfate) %	Drying method over P_2O_5 22 mm
gamma-poly-L-glu.	2	42.8	5.4				24 hrs 20°C
gamma-poly-L-glu.	4	44.8	5.7	10.2	0.0	0.0	24 hrs 20°C 5 hrs 110°C
gamma-poly-L-glu.	5	44.2	6.2	10.2	1.5	2.2	24 hrs 20°C 1 hr 100°C
gamma-poly-L-glu.	6	43.8	6.2		1.0	1.2	8 hrs 100°C
gamma-poly-D-glu.	7			10.4			24 hrs 20°C
gamma-poly-D-glu.	8	43.7	6.1	10.3	1.2	0.0	24 hrs 20°C
subtilis-polypeptide		43.8	5.5	10.1		0.0	24 hrs 20°C
Reported: $(C_{10}H_{14}O_6N_2 \cdot H_2O)_n$		43.6	5.85	10.2			
Reported: $(C_5H_7O_3N)_n$		46.5	5.5	10.8			

APPENDIX

Comparative serologic tests of synthetic polyglutamic acids of different constitution.

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By means of repeated intravenous inoculation of rabbits or horses with encapsulated anthrax bacilli killed by heat, immune sera are obtained, which show a precipitating reaction with solutions of natural polyglutamic acids up to a dilution of several millions (1, 2, appendix). By natural polyglutamic acids are meant the polyglutamic acids isolated from anthrax bacilli (B. anthracis)(anthrax-polypeptide) and the polyglutamic acid obtained from the nutrient of other aerobic, mesophilic sporebearers (for example, B. subtilis) (subtilis polypeptide). Both polypeptides demonstrate the properties of a hapten (semi-antigen), i.e. their parental dispensation is not followed by formation of an antibody (2,3, appendix).

As a supplement to the constitutional discovery of the anthrax-polypeptide and subtilis-polypeptide made by Bruckner et al, synthetic polyglutamic acids of various constitution were tested for their serologic activity. The test was accomplished with rabbit and horse sera containing anticapsular immune bodies; a subtilis-polypeptide preparation isolated by the original method (5, appendix) served as a comparative substance. Test results are listed in Tables 1 and 2. Table 1 contains results of experiments made by means of the ring test and the agar diffusion method. These data show that a horse serum containing anticapsular immune bodies is precipitated only by gamma-poly-D-glutamic acid and the mesoid gamma-polyglutamic acid. Polyglutamic acids of different constitution remained completely inactive, although this initial qualitative test was carried out with a moderate dilution of 1:1000.

It should be noted further in connection with the data of these tables that amino nitrogen values (ascertained after van Slyke) are given for the individual preparations, instead of their average molecular weight.

Table 1

Constitution of poly-glutamic acid	Method of synthesis (literature data)	Amino-N %	Serologic activity
(1) gamma-poly-D-glu.	4 (method 2)	1.5	+
(2) gamma-poly-D-glu.	4 (method 4)	0.65	+
(3) mesoid gamma-poly-glu.	4	2.9	+
(4) gamma-poly-L-glu.	4	0.36	+
(5) alpha-poly-L-glu.	6	0.14	-
(6) alpha-poly-D-glu.	6	0.12	-
(7) alpha gamma-poly-L-glu.	7	0.36	-
(8) alpha gamma-poly-D-glu.	7	0.43	-

Qualitative test of serologic activity of polyglutamic acids of different constitution with anti-anthrax horse immune sera. Dilution 10^3.

Table 2

Substance	Utilized immune serum	Precipitation titer (millions)	Amount of antibody precipitated from 1 ml serum (ug)
Subtilis-polypeptide	Rabbit-P	0.2	844
	Rabbit 46	2.5	1231
	Horse 971	2.5	945
	Horse-AS	3.0	1790
gamma-poly-D-glu.	Rabbit-P	no precipitation	
	Rabbit 46	2.5	819
	Horse 971	2.5	881
	Horse-AS	3.0	1740
mesoid gamma-poly-glu.	Horse-AS	2.0	1030

Table 2: Quantitative, comparative test of serological activity of gamma-poly-D-glutamic acid (Table 1, No. 1), mesoid gamma-poly-glutamic acid (Table 1, No. 3), and subtilis-polypeptide (amino-N 0.20%).

Table 2 shows results of the quantitative, comparative tests of serologic activity of subtilis-polypeptide isolated by the original method (5, appendix), of gamma-poly-D-glutamic acid (Table 1, No. 1) and of the mesoid gamma-poly-glutamic acid (Table 1, No. 3). It appears from these data that the synthetic gamma-poly-D-glutamic acid precipitates horse immune sera in as high a dilution as subtilis-polypeptide; the maximal amounts of separated antibody are also identical. The mesoid gamma-poly-glutamic acid showed a slightly weaker activity in horse serum than gamma-poly-D-glutamic acid. In rabbit serum 46 only a portion of its antibody content was precipitated by gamma-poly-D-glutamic acid. Oddly enough rabbit serum P, whose precipitation titer was fairly low, as determined with subtilis-polypeptide, did not show any precipitating reaction whatsoever with gamma-poly-D-glutamic acid. The fact that the antibody of this immune serum, produced 15 years ago and stored since, was still able to react specifically with gamma-poly-D-glutamic acid, was proved by its positive inhibition reaction. In determining this inhibition reaction, a precipitating reaction of subtilis-polypeptide and rabbit serum P, effected under optimal ratio conditions, served as a basis. This reaction was neutralized completely by 150 µg of gamma-poly-D-glutamic acid, partially by 30 µg. For the sake of completeness it should be noted here that none of the poly-glutamic acids marked as serologically inactive in Table 1 had any effect whatever on the precipitating reaction of rabbit serum P and subtilis-polypeptide, not even in the amount of 650 µg.

Aforementioned finer differences noted between the serologic action of subtilis-polypeptide, gamma-poly-D-glutamic acid and mesoid gamma-poly-glutamic

acid, may be caused by variations in average molecular weight or the dispersity of these polypeptides. These variations are easily recognized by means of Ouchterlony's agar diffusion method (8, appendix). While in the case of subtilis-polypeptide there occurred a less than 1 mm wide, very distinctly marked precipitation zone at a distance of 3 mm from the edge of the bore hole containing the immune serum, the turbidness caused by precipitation in the case of synthetic gamma-poly-D-glutamic acid appeared in the form of a 3 mm wide, inhomogeneous stripe, whose edge had advanced almost to the edge of the bore hole containing the immune serum. This difference indicates that subtilis-polypeptide is fairly homodisperse, while synthetic gamma-poly-D-glutamic acid represents a strongly polydisperse mixture whose major part consists of components having a lower molecular weight than the major components of subtilis-polypeptide. This difference should also explain the variable action of subtilis-polypeptide and gamma-poly-D-glutamic acid in respect to rabbit serum P. In this connection earlier observations (9,10, appendix) should be brought out to the effect that anthrax antibodies, depending on rabbit or horse passage, may react differently, even though their specificity is identical.

Experimental results summarized in Table 2 are to be supplemented by conclusions reached in testing of sera which had been absorbed by serologically active polyglutamic acids. Tests to this effect, carried out with horse serum AS, resulted in the following. Table 2 shows that synthetic gamma-poly-D-glutamic acid precipitates practically the same amount of antibody from horse serum AS as subtilis-polypeptide (1790 and 1740 µg; the small difference can be ascribed to a test error). The fluid, obtained after centrifugation of the precipitate, could not be brought to renewed precipitation either by addition of subtilis-polypeptide or of synthetic gamma-poly-D-glutamic acid. Mesoid gamma-poly-glutamic acid separated only 1030 µg of antibody, but after centri-

fugation of the fluid and addition of 25 µg of subtilis-polypeptide an additional 687 µg of antibody was precipitated, so that the total amounted to 1717 µg. However, once the antibody had been precipitated from the horse serum by the mesoid gamma-poly-glutamic acid under optimal ratio conditions of both solutions, additional mesoid gamma-poly-glutamic acid did not cause renewed precipitation from the centrifuged fluid.

In summing up it may be said that the results of serologic tests are in complete agreement with those of chemical experiments on the immune-specific, polypeptide-like hapten of the anthrax-subtilis bacilli group. Moreover, in our opinion these test results demonstrate a very interesting example of immune-specificity. It is clearly evident from the results of serologic tests that the reaction of the anticapsular anthrax antibody is structure-specific in respect to the constitution of polyglutamic acids, since of the latter only gamma-poly-glutamic acids of corresponding configuration show serologic activity, but not so alpha or alpha gamma-poly-glutamic acids. Furthermore this reaction is stereo-specific to a degree which has yet to be discovered, since of the gamma-poly-glutamic acids only gamma-poly-D-glutamic acid and mesoid gamma-poly-glutamic acid are serologically active, while gamma-poly-L-glutamic acid shows no serologic activity. Whether or not the weak serologic activity of mesoid gamma-poly-glutamic acid is really to be ascribed to its own constitution, i.e. to the presence of 50% of gamma-L-glutamic acid residues, must be reaffirmed by additional tests, since the substance tested here had a much higher content of amino nitrogen (i.e. probably a much lower molecular weight) than gamma-poly-D-glutamic acid.

Methods.

The subtilis-polypeptide used for comparison was isolated in accordance with the original method (5, appendix).

The rabbit immune sera were prepared by Tomcsik's and Ivanovics' method. One of these (designation: P) had been produced in 1940 and stored since that time, while the other (46) was made just prior to the start of the experiments. The horse immune sera were prepared by the Institute of Vaccine Production ("Phylaxia", Budapest) in 1940, and this by inoculation of horses with live, avirulent, encapsulating anthrax bacilli.

In order to determine the precipitation titer, dilution series according to the principle of double dilution were prepared from the solution of the substance to be tested, and 0.2 ml each of these samples were mixed with 0.2 ml each of the serum, which had been diluted 1:2. Incubation: 48 hrs at 4°C.

The serologic inhibition reactions were conducted by a method similar to that employed in determining the precipitation titer. Samples of subtilis-polypeptide solution diluted to the degree of optimal precipitation were mixed first with varying amounts of polyglutamic acids to be tested, then with the serum.

The quantitative precipitation tests were carried out as follows: In a cooled centrifugal tube, 1 ml of serum and 25 µg of the substance to be tested were mixed in 1 ml of solution, the mixture was stored in the refrigerator for 48 hours, then the precipitate was centrifuged off and washed twice by soaking in 5 ml of ice cold brine each, followed by repeated centrifugation. The precipitate thus obtained was decomposed according to Cleghorn's and Jendrassik's method (11, appendix) and its nitrogen content was ascertained with Nessler's reagent by means of three parallel determinations. Product nitrogen µg 6.25 was designated as the quantity of precipitated antibody.

The diffusion of the polypeptides was tested by Ouchterlony's method (8, appendix) in agar gel prepared from buffered brine containing 1% agar. The edges of the two bore holes containing the serum and the solution of the substance to be tested were 9 mm apart.